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Targeting notch3 with CRISPR-Cas9 technology in zebrafish retinal degeneration models

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Targeting *notch3* with CRISPR-Cas9 technology in zebrafish retinal degeneration models

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Introduction

Teleost fish such as zebrafish (*Danio rerio*) have shown the ability to regenerate damaged photoreceptors due to the robust activity of Müller glia [1]. Interestingly, the Müller glia in retinal disease model zebrafish such as *bbs2*, *cep290*, and *eys* do not respond like those in normal zebrafish, making these genotypes a good model to study treatment options in humans [2, 3, 4]. The Notch signaling pathway is a highly conserved cell signaling pathway in many organisms that plays a role in determining cell fates in developmental processes [5]. Specifically, the *notch3* gene has been studied and shown to have effects on Müller glia proliferation [6,7]. In the zebrafish retina, Notch signaling has been determined to be necessary to inhibit the differentiation and proliferation of Müller glia cells [6]. What is unknown is how retinal disease model zebrafish respond to *Notch* signaling targets. In the current study, the embryos of each disease model line (*bbs2, cep290,* and *eys*) were injected with two different CRISPRs separately, targeting exon 2 and exon 4 of the *notch3* gene, and retinal tissue sections of 4–6-month-old zebrafish were stained for PCNA to quantify retinal regeneration.

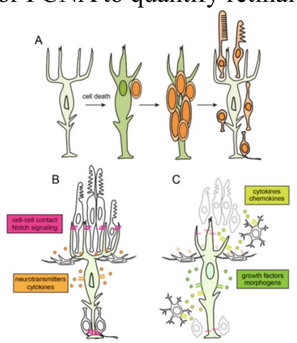

Figure 1. Schematic drawing of Müller glia regeneration [8].

Materials and Methods

- Guide RNAs were designed with the Alt-R CRISPT HDR Design Tool on the Integrated DNA Technologies website (Integrated DNA Technologies).
- Two primer sets were designed (Pick Primers) to target and amplify each of the designed gRNAs. These primers were ordered and tested in heterozygous zebrafish by using Polymerase Chain Reaction (PCR) and High-Resolution Melting Analysis (HRMA) techniques.
- Breedings between male and female zebrafish of each of the three genotypes (*bbs, cep290,* and *eys*) were set up daily for several weeks. Breeding tanks were set up to collect eggs. 1 nL of the CRISPR mixture was injected into the 1-cell stage embryos and uninjected eggs were collected for negative controls.
- Embryos were monitored for evidence of lethality over the first 5 days of life. All surviving larvae at this point were placed in separate tanks.
- 4–6-month-old fish were euthanized with ice water and the eyes were collected to be sectioned with a cryostat machine.
- Retinal tissue sections (10 µm) were stained for PCNA and DAPI. PCNA + cells were counted, and statistical analysis was used to compare groupings.

Results

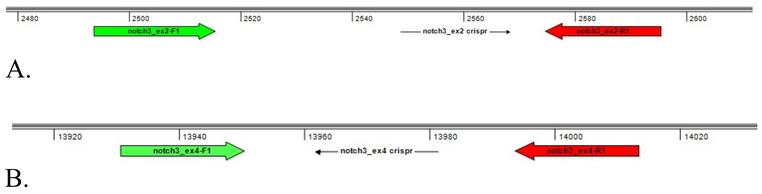

Figure 2. **Generation of zebrafish *notch3* CRISPRs and primers.** Sequences for *notch3* exon 2 and exon 4 were generated. Location of *notch3* exon 2 **(A)** and exon 4 **(B)** in the *notch3* gene of zebrafish.

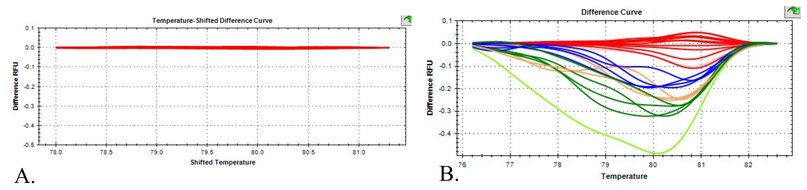

Figure 3. **Testing CRISPR activity in *bbs2*, *cep290*, and *eys* heterozygous zebrafish DNA**. *notch3* exon 2 primers run with 12 uninjected samples on HRMA in *cep290* **(A).** *notch3* exon 2 injected into 12 samples and 12 uninjected controls run on HRMA in *cep290* **(B)**.

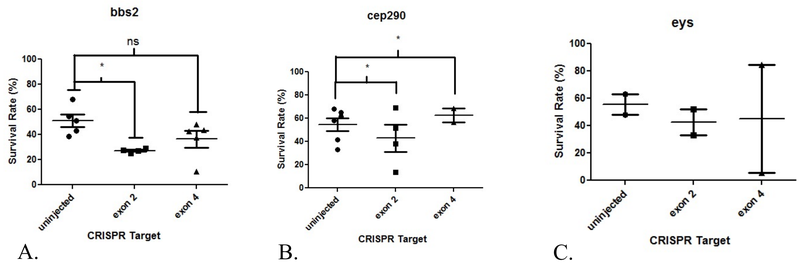

Figure 4. **Comparing survival rates in uninjected and injected zebrafish.** The survival of the zebrafish in separate clutches over the first 5 dpf was compared between uninjected and injected samples within *bbs2* **(A),** *cep290* **(B),** and *eys* **(C)** zebrafish. Data are presented as the mean *P <0.05 vs. uninjected groups (one-way ANOVA).

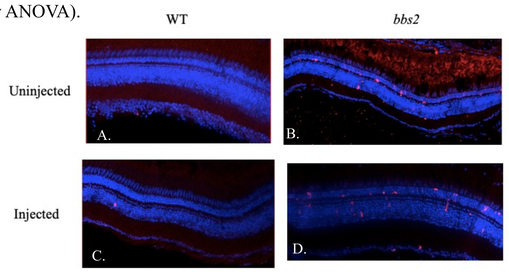

Figure 5. **Analyzing PCNA in retinal sections of uninjected and injected 4 mpf *bbs2* animals.** 20 µm retinal sections of uninjected and injected zebrafish were stained for PCNA (red) and DAPI (blue). Uninjected WT (A), uninjected *bbs2* MT (B), *notch3* injected WT (C), *notch3* injected *bbs2* MT (D) retinal sections were studied.

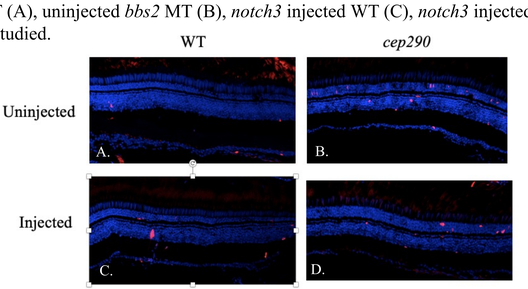

Figure 6. **Analyzing PCNA in retinal sections of uninjected and injected 6 mpf *cep290* animals.** 20 µm retinal sections of uninjected and injected zebrafish were stained for PCNA (red) and DAPI (blue).. Uninjected WT (A), uninjected *cep290* MT (B), *notch3* injected WT (C), *notch3* injected *cep290* MT (D) retinal sections were studied.

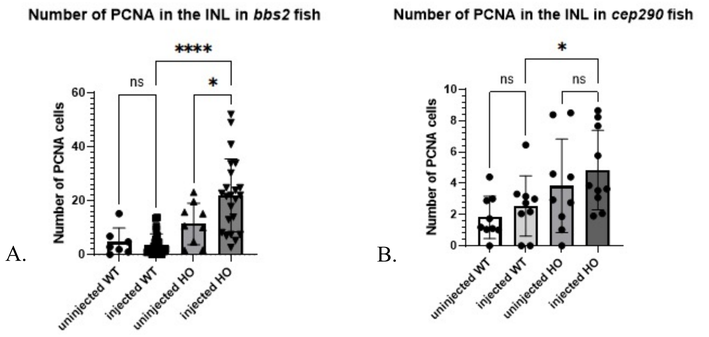

Figure 7. **Number of PCNA cells in the INL in uninjected and injected WT and MTs in *bbs2* (A) and *cep290* (B) fish.** Data are presented as the mean *P <0.05 between the above groups (one-way ANOVA).

Discussion

Two CRISPRs were successfully generated with targets for exons 2 and 4 of the *notch3* gene. (Figure 2). The DNA of the injected zebrafish produced deviated melt curves compared to the DNA of the uninjected zebrafish, which confirmed that they were effectively disrupting the gene activity in the respective exon (Figure 3). There were some significant differences seen in the survival of the zebrafish (Figure 4), but hundreds of zebrafish were still placed in tanks for development. Eyes were sectioned, and retinal tissue was stained for PCNA (Figures 5 and 6), and numbers were quantified. The significant increase in the number of PCNA positive cells in the INL of injected *bbs2* and *cep290* mutant fish compared to injected wildtype shows that the CRISPRs effectively triggered Müller glia proliferation, in a much larger quantity in *bbs2* fish (Figure 7a). The significant increase in the number of PCNA positive cells of injected *bbs2* mutants compared to injected wildtype confirms that there is an increase in proliferation of Müller glia in injected mutants, confirming the CRIPSR triggers regeneration, but no difference in cep290 zebrafish suggests the regeneration was not as robust in this genetic line (Figure 7b). These results suggest that knockdown of *notch3* could be a novel way to treat retinal diseases.

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